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14. ABSTRACT

We have established that PKC isozymes are important regulators of the synthesis and release of death factors in prostate cancer cells. In response to phorbol ester treatment, LNCaP cells promote the release of TNFα, TRAIL, and MCP-1/CCL2. Our recent studies revealed that all three factors contribute to the apoptotic effect of phorbol esters in prostate cancer cells. We established that PKCα and PKCδ mediate the sensitizing effect of CCL2. We also found that unlike PKCδ, PKCε has a pro-survival role in LNCaP cells. It is unclear yet whether PKCε modulates the synthesis and/or release of death factors. Finally, we established that androgens modulate the synthesis and release of pro-apoptotic cytokines from LNCaP cells. Androgen depletion or androgen receptor RNAi markedly impacted the apoptotic effect of death factors as well as the activation of p38 MAPK and JNK pathways.

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INTRODUCTION

We have been focusing our research on the roles of cytokines as modulators or effectors of protein kinase C (PKC) isozymes in prostate cancer. A key objective of the research supported by DOD is to elucidate the molecular mechanisms that mediate phorbol ester-induced apoptosis in prostate cancer cells. It is well established that phorbol esters activate PKC, a family of 10 different isozymes that has been classified into "classical" or calcium dependent PKCs (cPKCs α, βI, βII, and γ), "novel" or calcium-independent (nPKCs δ, ε, η, and θ), and "atypical" (aPKCs ζ and λ/ι). Phorbol esters only bind to and activate cPKCs and nPKCs, and they mimic the action of the lipid second messenger diacylglycerol (DAG), thus used extensively to mimic the effects of DAG generated upon receptor activation. PKC isozymes have been initially viewed as tumor promoting kinases. However, subsequent studies established that they can also activate growth inhibitory pathways in many cell types. Various members of the PKC family have been implicated in malignant transformation, the control of cell cycle progression, survival, and apoptosis (1, 2).

We have previously established that treatment of androgen-dependent LNCaP prostate cancer cells with phorbol esters triggers an apoptotic response, and that this effect is primarily mediated by PKCδ. For example, phorbol 12-myristate 13-acetate (PMA) induces apoptosis in androgen-dependent LNCaP prostate cancer cells, and this effect can be abolished by either pharmacological means, such as with the "pan"-PKC inhibitor GF109203X or the PKCδ inhibitor rottlerin, or by depleting PKCδ using RNAi (3-5). The roles for other phorbol ester-responsive PKCs are less clear, but a pro-apoptotic role for PKCα in LNCaP cells has been also established (4, 6). PKCε, the other phorbol ester-responsive PKC expressed in LNCaP cells, plays a role in cell survival in various models (7-10). A thorough mechanistic analysis of the signaling players in phorbol ester-induced apoptosis in LNCaP cells revealed key roles for p38 and JNK cascades (5). Studies from our laboratory have determined that the activation of pro-apoptotic pathways by PMA is caused by death factors released from LNCaP cells in response to phorbol ester treatment. When we collected conditioned medium (CM) from LNCaP cells treated with PMA (CM-PMA) and assayed for its ability to induce apoptosis, we found that it caused a marked apoptotic response. On the other hand, CM collected from vehicle-treated cells (CM-Veh) was ineffective. A more extensive analysis revealed that this autocrine effect is mediated primarily by the cytokines TNFα and TRAIL. PKCδ is implicated both in the release of the death factors as well as a downstream effector of death receptors (11).

A main goal of the funded DOD application was to identify additional factors that contribute to the autocrine effect, as TNFα and TRAIL alone or in combination were not sufficient to promote a full apoptotic response in LNCaP cells similar to that observed in response to PMA treatment. Our preliminary studies identified a potential candidate: MCP-1 (Monocyte Chemoattractant Protein-1 (also called CCL2) and suggested that other cytokines were also induced in response to PMA, such as IL-8 (interleukin-8). Data from our laboratory showed that MCP-1/CCL2 potentiates the apoptotic effects of TNFα and TRAIL in LNCaP cells. A main goal is to establish the relationship between individual PKC isozymes and cytokines as mediators of phorbol ester-induced apoptotic responses in prostate cancer cells. We also aimed at establishing a potential role for androgens as modulators of these responses.

BODY

1. A neutralizing antibody against MCP-1/CCL2 inhibits PMA-induced apoptosis. In order to determine whether CCL2 contributes to the apoptotic effect of the CM-PMA in LNCaP cells, we used a CCL2 neutralizing antibody. This antibody was added at different concentrations (0.3-10 μg/ml) to the CM-PMA (conditioned medium from a LNCaP culture collected 24 h after 100 nM PMA treatment). CM-PMA was then added to LNCaP cells and apoptosis determined 24 h later. As shown in Fig. 1, this antibody partially blocked the apoptotic effect of CM-PMA in LNCaP cells. Approximately 41 ± 12 % inhibition was observed at 10 ng/ml. On the other hand, a control antibody (mouse IgG, 10 ng/ml) did not have any significant effect. This suggests that CCL2 secreted to the CM contributes to the apoptotic effect of phorbol esters in LNCaP cells.

2. A neutralizing antibody against MCP-1/CCL2 enhances the inhibitory effect of anti-TNFα and anti-TRAIL neutralizing antibodies on PMA-induced apoptosis. Next, we determined whether blocking antibodies against TNFα and/or TRAIL had also an effect on the apoptotic effect of the phorbol ester. CM-PMA was collected, as described above, incubated with blocking antibodies against TNFα (1 ng/ml) and TRAIL (0.5 ng/ml), and then added to LNCaP cells. Apoptosis was determined 24 h later. As shown in Fig. 2, apoptosis was reduced (50 ± 3 % inhibition) when CM-PMA was incubated with TNFα/TRAIL neutralizing antibodies. Interestingly, the addition of a CCL2 blocking antibody (1 μg/ml) significantly enhanced this effect (69 ± 2 % inhibition). These results strongly suggest that TNFα, TRAIL and CCL2 secreted to the medium in response to PMA contribute to the pro-apoptotic effect of CM-PMA. These results also validate preliminary results presented in our original application in which we found that the apoptotic effect of PMA in LNCaP cells was partially inhibited when the CCL2 receptor (CCR2) was depleted using RNAi.

3. PKCα and PKCδ mediate the sensitizing effect of MCP-1/CCL2. We have previously found that the apoptotic effect of CM-PMA was impaired by PKC inhibition, suggesting that PKC isozymes were involved not only in factor release but also as downstream effectors of these factors (11). Not surprisingly, the apoptotic effect of TNFα/TRAIL in the presence of CCL2 was inhibited by 59 ± 5 % by the pan-PKC inhibitor GF109203X (18 ± 1 % apoptosis in the absence of GF109203X *vs*. 7 ± 1 % in the presence of 5 μM GF109203X, n=3, $p < 0.05$).

We speculated that PKCα and/or PKCδ play a role as mediators of the CCL2 effect. To address this issue, LNCaP cells were transfected with specific dsRNAs for either PKCα or PKCδ isozymes, or a control dsRNA. Significant depletion was achieved for PKCα and PKCδ 48 h after transfection (67 ± 14% and 86 ± 11% depletion, respectively (Fig. 3). PKCα RNAi did not affect the apoptotic response of TNFα/TRAIL, although a small but still significant reduction was observed in PKCδ-depleted LNCaP cells. Similar results were observed with a second set of RNAi duplexes (data not shown). However, either PKCα or PKCδ RNAi were able to block the sensitizing effect of CCL2 (Fig. 3), suggesting not only a requirement for both PKCs, but also that there are no compensatory effects when only one of the PKCs was depleted using RNAi.

4. Studies on PKC translocation by cytokines. One of the goals in our proposal was to determine whether PKCs can be translocated by cytokines in LNCaP prostate cancer cells. We have previously reported that phorbol esters promote the redistribution of PKCs to membranes in

LNCaP cells, with different localizations for discrete PKC isozymes (4). We treated LNCaP cells with TNFα (1 ng/ml) ± TRAIL (0.5 ng/ml), or PMA (100 nM) and determined translocation by ultracentrifugation of cell extracts, followed by Western blot. Most PKCα and PKCδ was found in the soluble (cytosolic) fraction before stimulation. In response to PMA (10-1000 nM), a concentration-dependent translocation to the particulate fraction is normally observed for either PKC. However, we could not detect any significant translocation of PKCs by the death factors (data not shown). These results do not completely rule out translocation of PKCs, as it is our experience, at least with growth factors, that translocation is transient and difficult to detect unless a stable phorbol ester analog such as PMA is used in these assays. We are currently evaluating whether translocation in LNCaP cells by death factors could be addressed by microscopy using GFP-fused PKCs, as shown in Ref. 4.

5. PKCε has a pro-survival role in LNCaP cells. PKCδ and PKCε isozymes in most cases have a "yin-yang" relationship and mediate opposite responses (1). This was originally described in NIH 3T3 fibroblasts, in which PKCδ overexpression leads to growth arrest while PKCε overexpresssion transforms these cells and confers tumorigenic properties when inoculated into nude mice, thereby suggesting a potential role for PKCε as an oncogene (12). PKCε can signal to mitogenesis via Raf/MEK/ERK and cyclin D1 induction (13-15) and has been also linked to cancer cell survival through the activation of Akt, Bax, or other pro-survival molecules (7-10). It is important to note that the balance in PKC isozyme expression is markedly altered in human prostate tumors, potentially reflecting their involvement in the etiology and progression of the disease. Numerous studies have found that PKCε expression is up-regulated in prostate cancer specimens (16-19). We speculated that PKCε may oppose PKCδ apoptotic responses in prostate cancer cells (*i.e.* apoptosis *vs.* survival). Whether PKCε modulates the secretion of cytokines from LNCaP in response to PMA stimulation is unknown.

In the first set of experiments, to determine if PKCε plays a role in prostate cancer cell survival, we delivered a specific PKCε RNAi duplex into LNCaP cells. We achieved > 90% depletion 48 h after transfection, without affecting expression of other PKCs. Notably, apoptosis induced by PMA was significantly enhanced in PKCε-depleted LNCaP cells, while PKCδ RNAi totally blocked the response (Fig. 4A). The effect was validated with additional RNAi duplexes (data not shown). Likewise, adenoviral delivery of a dominant-negative PKCε mutant potentiates PMA-induced apoptosis (Fig. 4B). Thus, PKCδ and PKCε mediate opposite responses in LNCaP cells. Remarkably, androgen-independent DU145 cells, which are resistant to PMA-induced apoptosis, are sensitized to PMA-induced cell death when subject to PKCε RNAi (Fig. 4C). The effect of PKCε RNAi was also observed in the LNCaP variant C4 (Fig. 4C). Thus, PKCε protects against cell death in prostate cancer cells.

To further validate this conclusion, we overexpressed PKCε in LNCaP cells using an adenovirus. Fig. 5 shows that PKCε overexpression protects LNCaP cells against apoptosis induced by PMA. This effect is proportional to the expression levels achieved by varying multiplicities of infection. This further confirms a pro-survival role for PKCε in prostate cancer cells.

It would be interesting now to determine whether PKCε RNAi depletion potentiates the apoptotic effect of CM-PMA as well as apoptosis induced by TNFα/TRAIL/CCL2. Our hypothesis is that PKCε depletion sensitizes LNCaP cells to the apoptotic effect of death factors

released in response to PMA. It would be also interesting to determine whether the release of death factors is modulated by PKCε.

7. CCL2 mRNA up-regulation by PMA is also observed in a microarray study of PMA-regulated genes. As phorbol esters are strong inducers of gene expression, including genes for pro-apoptotic cytokines, we speculated that apoptosis induced by PMA may have a transcriptional component. Indeed, when LNCaP cells were pre-treated with the protein synthesis inhibitor cycloheximide, the apoptotic response of PMA was reduced by 44% ($p<0.05$), suggesting the involvement of newly synthesized proteins and a potential transcriptional component. We therefore decided to study changes in gene expression using Affymetrix microarrays. These studies were carried out at the UPenn Microarray Facility, directed by Dr. Don Baldwin. We studied the dynamics of changes in gene expression at 4, 8, 12, and 24 h after PMA (Fig. 6A). We found 4,949 genes regulated by PMA (> 2-fold change) and identified early and late genes (Fig. 6B). A gene ontology analysis revealed a large number of apoptotic/survival, proliferation, oncogenesis, and transcription genes (Fig. 6C).

Of particular interest for the work funded by DOD, the microarray study revealed that PMA induces a strong up-regulation of cytokines. Remarkably, we found CCL2 as one of the highest PMA-up-regulated genes (> 1,000-fold change), which validates results from our previous studies. We also found a remarkable up-regulation of other cytokines and chemokines, including IL-8, CCL8, CLC20, CXCL10, CXCL11, and Fas, as well as receptors such as CXCR4, TNFRSF11, TNFRSF12, and IL6R.

To our knowledge there are no studies on gene expression regulated by specific PKC isozymes in prostate cancer cells or any other cell type. We are now carrying on the first PKC isozyme-specific microarray analysis. For this study we treated with PMA (100 nM, 4 h) LNCaP cells subject to RNAi depletion for PKCα, δ, or ε (2 different duplexes/PKC). We aim to identify for the first time isozyme-specific regulated genes, and we hope that these results will provide important information on the isozyme-specific regulation of cytokines implicated in phorbol ester-induced apoptosis as well as other key elements involved in this response.

8. Androgens modulate the release of cytokines from LNCaP cells. As LNCaP cells are androgen-dependent and we recently reported that androgens regulate the expression of PKCδ at a transcriptional level (20), we reasoned that in LNCaP cells growing in the absence of androgens the ability of phorbol esters to promote the autocrine secretion of death factors should be affected.

In the previous funding cycle we provided preliminary data showing that androgens modulate the PMA-mediated autocrine apoptotic response. In those experiments we showed that CM-PMA collected from LNCaP cells growing either in normal medium or in steroid-depleted medium (charcoal-treated) have different potency for inducing apoptosis in LNCaP cells. Indeed, the apoptogenic activity of CM-PMA collected from LNCaP cells growing in steroid-depleted medium was markedly reduced compared to that collected from cells growing in normal medium. Addition of the synthetic androgen R1881 to the steroid-depleted medium restores the full apoptotic response of CM-PMA. We speculated that the secretion of apoptotic factors from LNCaP cells is regulated by androgens, and preliminary data presented before showed that the induction of TNFα mRNA by PMA was markedly reduced when LNCaP cells were grown in steroid-depleted medium, an effect restored by the addition of R1881 to the steroid-depleted medium.

In a previous study we showed PMA promotes a marked release of TNFα from LNCaP cells, as determined by ELISA. This effect was inhibited by GF109203X (a “pan” PKC

inhibitor), rottlerin (a PKCδ inhibitor), or PKCδ RNAi (11). Interestingly, we now show that the release of TNFα by PMA was blunted in LNCaP cells growing in steroid-depleted medium. This effect was restored by addition of R1881 to the medium (Fig. 7A). TRAIL levels in CM-PMA were also higher than in CM-Veh, as determined by ELISA (Fig. 7B). Moreover, TRAIL levels were significantly lower in CM-PMA collected from LNCaP cells growing in steroid-depleted medium relative to CM-PMA collected from cells growing in normal medium. As with TNFα, when the steroid-depleted medium was supplemented with R1881, PMA was able to cause a full release of TRAIL. We have not yet measured the effect of androgen depletion on CCL2 levels.

9. Androgen receptor (AR) depletion impairs the PMA-induced autocrine loop. To further establish the relevance of our findings, we analyzed the effect of AR depletion on the autocrine secretion of death factors. We have previously determined that AR knock-down using RNAi abrogates the apoptotic effect of PMA in LNCaP cells (20). Delivery of a specific AR RNAi duplex into LNCaP cells caused a significant reduction in AR levels as well as PKCδ down-regulation (Fig. 8A). Interestingly, CM-PMA collected from AR-depleted LNCaP cells had significantly lower apoptotic activity compared to that from cells transfected with a control RNAi duplex (Fig. 8B). The partial inhibition may be a consequence of the incomplete AR and PKCδ depletion achieved in these experiments. The ability of PMA to induce TNFα and TRAIL mRNA was markedly diminished in LNCaP cells subject to AR knock-down (Fig. 8C and 8D). Moreover, in AR-depleted LNCaP cells, the release of TNFα and TRAIL by PMA was impaired (Fig. 8E and 8F). We have not yet measured the effect of AR depletion on CCL2 levels. Collectively, our data implicate androgens as key modulators of cytokine release and programmed cell death in response to PKC activation.

9. Androgen depletion and AR RNAi inhibit the activation of p38 MAPK and JNK by factors released in response to PMA activation. Since the autocrine-mediated apoptotic effect of phorbol esters in LNCaP cells is mediated by the p38 MAPK and JNK cascades (11), we decided to determine whether androgen depletion impacts on the activation of these signaling pathways. Addition of CM-PMA to LNCaP cells caused a time-dependent activation of p38 MAPK and JNK. Interestingly, CM-PMA collected from AR-depleted LNCaP cells has a reduced efficacy to activate p38 MAPK and JNK (Fig. 9A). Likewise, activation of p38 MAPK and JNK by CM-PMA collected from steroid-depleted LNCaP cells was greatly diminished compared to that caused by CM-PMA collected from cells growing in normal medium. This effect was rescued by the addition of R1881 to the medium (Fig. 9B).

KEY RESEARCH ACOMPLISHMENTS

- We established that a neutralizing antibody against CCL2 impairs CM-PMA-induced apoptosis in LNCaP cells.
- We found that addition of anti-TNFα, anti-TRAIL, and anti-CCL2 antibodies to CM-PMA, impairs its apoptotic effect in LNCaP cells. This suggests that multiple cytokines/chemokines mediate phorbol ester-induced apoptosis in LNCaP cells.
- We found that PKCα and PKCδ mediate the sensitizing effect of CCL2. PKCα RNAi did not affect the apotoptic response of TNFα/TRAIL, and a small but still significant reduction was observed in PKCδ-depleted LNCaP cells. Either PKCα or PKCδ RNAi

- We initiated studies on PKC isozyme translocation by cytokines.
- We established that unlike PKCδ, PKCε has a pro-survival role in LNCaP cells. PKCε RNAi depletion in LNCaP cells or delivery of a dominant-negative PKCε mutant significantly enhanced PMA-induced apoptosis in LNCaP cells, and PKCε RNAi depletion sensitized androgen-independent DU145 cells for apoptosis. On the other hand, PKCε overexpression protected LNCaP cells against apoptosis induced by the phorbol ester. These results indicate that PKCε has a pro-survival role in prostate cancer cells.
- A microarray analysis confirmed that CCL2 mRNA is markedly up-regulated by PMA. In addition, this study also found that the expression of other cytokines and chemokines, as well as their receptors is modulated by PMA in LNCaP cells.
- We established that androgens modulate the synthesis and release of pro-apoptotic cytokines from LNCaP cells. Similar results were observed in LNCaP cells in which AR has been depleted using RNAi.
- CM-PMA collected from LNCaP cells growing in androgen-depleted medium or subject to AR RNAi depletion poorly activate p38 MAPK and JNK pathways. Therefore, our data implicate androgens as key modulators of cytokine release and apoptosis in response to PKC activation in prostate cancer cells.

REPORTABLE OUTCOMES

Xiao L, Caino MC, von Burstin VA, Oliva JL, Kazanietz MG. Phorbol ester-induced apoptosis and senescence in cancer cell models. Methods Enzymol. 446: 123-139 (2008).

Xiao L, Gonzalez-Guerrico A, Kazanietz MG. PKC-mediated secretion of death factors in LNCaP prostate cancer cells is regulated by androgens. Mol Carcinog. 2008 Aug 28. [Epub ahead of print].

CONCLUSIONS

We clearly established that phorbol ester-induced apoptosis in LNCaP cells is mediated by the release of death factors (TNFα/TRAIL), and that MCP-1/CCL2 released from LNCaP cells contributes to the apoptotic effect. Phorbol esters control the expression and release of these cytokines/chemokines. PKCδ is a major player in the release of these factors as well as an effector of the apoptotic response. Our studies also revealed a pro-survival role for PKCε in LNCaP cells. Whether this relates to a modulation of the autocrine secretion of death factors and CCL2, or whether PKCε modulates apoptotic signaling by these factors, remain to be elucidated. Our studies also established an important role for androgens in the control of phorbol ester-induced apoptosis in LNCaP cells. Androgens modulate the secretion of cytokines/chemokines, and this ultimately impacts on the apoptotic response of the phorbol ester. Our studies not only have the potential to uncover important novel apoptotic mechanisms in prostate cancer cells, but may also have significant therapeutic implications.

REFERENCES

1. Griner, E.M., and Kazanietz, M.G. Protein kinase C and other diacylglycerol effectors in cancer. Nature Reviews Cancer 7: 281-294 (2007).
2. Newton, A.C. Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. Chem. Rev. 101: 2353-2364 (2001).
3. Fujii, T., García-Bermejo, M.L., Bernabó, J.L., Caamano, J., Ohba, M., Kuroki, T., Li, L., Yuspa, S.H., and Kazanietz, M.G. Involvement of PKCδ in phorbol ester-induced apoptosis in LNCaP prostate cancer cells. Lack of proteolytic cleavage of PKCδ. J. Biol. Chem. 275: 7574-7582 (2000).
4. Garcia-Bermejo, M.L., Leskow, F.C., Fujii, T., Wang, Q., Blumberg, P.M., Ohba, M., Kuroki, T., Han, K.C., Lee, J., Marquez, V.E., and Kazanietz, M.G. Diacylglycerol (DAG)-lactones, a new class of protein kinase C (PKC) agonists, induce apoptosis in LNCaP prostate cancer cells by selective activation of PKCalpha. J. Biol. Chem. 277: 645-655 (2002).
5. Tanaka, Y., Gavrielides, M.V., Mitsuuchi, Y., Fujii, T., and Kazanietz, M.G. Protein kinase C promotes apoptosis in LNCaP prostate cancer cells through activation of p38 MAPK and inhibition of the Akt survival pathway. J. Biol. Chem. 278: 33753-33762 (2003).
6. Powell CT, Brittis NJ, Stec D, Hug H, Heston WD, Fair WR.Persistent membrane translocation of protein kinase C alpha during 12-O-tetradecanoylphorbol-13-acetate-induced apoptosis of LNCaP human prostate cancer cells. Cell Growth Differ. 7: 419-428 (1996).
7. McJilton MA, Van Sikes C, Wescott GG, Wu D, Foreman TL, Gregory CW, Weidner DA, Harris Ford O, Morgan Lasater A, Mohler JL, Terrian DM. Protein kinase Cepsilon interacts with Bax and promotes survival of human prostate cancer cells. Oncogene 22: 7958-7968 (2003).
8. Lu D, Huang J, Basu A.Protein kinase Cepsilon activates protein kinase B/Akt via DNA-PK to protect against tumor necrosis factor-alpha-induced cell death. J. Biol. Chem. 281: 22799-22807 (2006).
9. Okhrimenko H, Lu W, Xiang C, Hamburger N, Kazimirsky G, Brodie C.Protein kinase C-epsilon regulates the apoptosis and survival of glioma cells. Cancer Res. 65: 7301-7309 (2005).
10. Liu H, Qiu Y, Xiao L, Dong F.Involvement of protein kinase Cepsilon in the negative regulation of Akt activation stimulated by granulocyte colony-stimulating factor. J. Immunol. 176: 2407-2413 (2006).
11. Gonzalez-Guerrico, A., and Kazanietz, M.G. Phorbol ester-induced apoptosis in prostate cancer cells via autocrine activation of the extrinsic apoptotic cascade. A key role for protein kinase Cdelta. J. Biol. Chem. 280: 38982-3899 (2005).
12. Mischak H, Goodnight JA, Kolch W, Martiny-Baron G, Schaechtle C, Kazanietz MG, Blumberg PM, Pierce JH, Mushinski JF.Overexpression of protein kinase C-delta and -epsilon in NIH 3T3 cells induces opposite effects on growth, morphology, anchorage dependence, and tumorigenicity. J. Biol. Chem. 268: 6090-6096 (1993).
13. Slupsky JR, Kamiguti AS, Harris RJ, Cawley JC, Zuzel M. Central role of protein kinase Cepsilon in constitutive activation of ERK1/2 and Rac1 in the malignant cells of hairy cell leukemia. Am. J. Pathol. 170: 745-754 (2007).
14. Kampfer S, Windegger M, Hochholdinger F, Schwaiger W, Pestell RG, Baier G, Grunicke HH, Uberall F.Protein kinase C isoforms involved in the transcriptional activation of cyclin D1 by transforming Ha-Ras. J. Biol. Chem. 276: 42834-43842 (2001).
15. Schönwasser DC, Marais RM, Marshall CJ, Parker PJ. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. Mol. Cell. Biol. 18: 790-798 (1998).

16. Cornford P, Evans J, Dodson A, Parsons K, Woolfenden A, Neoptolemos J, Foster CS. Protein kinase C isoenzyme patterns characteristically modulated in early prostate cancer. Am J Pathol. 154: 137-144 (1999).
17. Koren R, Ben Meir D, Langzam L, Dekel Y, Konichezky M, Baniel J, Livne PM, Gal R, Sampson SR.Expression of protein kinase C isoenzymes in benign hyperplasia and carcinoma of prostate. Oncol Rep. 11: 321-326 (2004).
18. Aziz MH, Manoharan HT, Church DR, Dreckschmidt NE, Zhong W, Oberley TD, Wilding G, Verma AK. Protein kinase Cepsilon interacts with signal transducers and activators of transcription 3 (Stat3), phosphorylates Stat3Ser727, and regulates its constitutive activation in prostate cancer. Cancer Res. 67: 8828-8838 (2007).
19. Wu D, Foreman TL, Gregory CW, McJilton MA, Wescott GG, Ford OH, Alvey RF, Mohler JL, Terrian DM.Protein kinase cepsilon has the potential to advance the recurrence of human prostate cancer. Cancer Res. 62: 2423-2429 (2002).
20. Gavrielides, M.V., Gonzalez-Guerrico, A., Riobo, N.A., and Kazanietz, M.G. Androgens regulate PKCδ transcription and modulate its apoptotic function in prostate cancer cells. Cancer Res. 66: 11792-11801 (2006).

APPENDIX

9 figures and the corresponding figure legends.

FIGURE LEGENDS

Figure 1. CCL2 in the conditioned medium from PMA-treated cells (CM-PMA) has pro-apoptotic activity. LNCaP cells at approximately 75% confluence were treated with 100 nM PMA, and CM-PMA was collected 24 h later. Another culture of LNCaP cells was treated with CM-PMA, either in the presence or absence of increasing concentrations of a CCL2 neutralizing antibody or normal rabbit IgG. Incubation of the CM with the antibodies was carried out for 30 min before adding the CM to the cells. The incidence of apoptosis was determined 24 h later. Results are presented as mean ± S.D. of one experiment made in triplicate, and it is representative of 3 independent experiments.

Figure 2. Effect of combined neutralizing antibodies on CM-PMA-induced apoptosis. LNCaP cells were treated with CM-PMA, either in the presence or absence of different neutralizing antibodies (1 μg/ml of anti-CCL2 antibody, 1μg/ml of anti-TNFα antibody; 0.5 μg/ml of anti-TRAIL antibody) or normal IgG (1 μg/ml anti-mouse and 1.5 μg/ml anti-rabbit). CM-PMA was incubated with the antibodies 30 min before added to the cells. Percentage of apoptotic cells was determined by DAPI staining 24 h after treatment. Results are presented as mean ± S.D. of one experiment made in triplicate, and it is representative of 3 independent experiments.

Figure 3. Both PKCα and PKCδ mediate the sensitizing effect of CCL2. LNCaP cells were transfected with specific RNAi duplexes for PKCα or PKCδ or a control duplex. Forty eight h later cells were subject to Western blot using specific anti-PKC antibodies (*Panel A*) or treated with 10 ng/ml of TNFα and 20 ng/ml of TRAIL either in the absence or presence of 10 ng/ml of CCL2. The incidence of apoptosis was determined 24 h later by DAPI staining (*Panel B*). Data are presented as mean ± S.E. of 3 independent experiments carried out in triplicate ($n = 3$).

Figure 4. Opposite roles of PKCδ and PKCε in LNCaP cells. *Panel A.* LNCaP cells were transfected with PKCδ, PKCε, or control (*C*) siRNAi duplexes using the Amaxa Nucleofector. After 48 h, cells were treated with PMA (100 nM, 1 h), and the incidence of apoptosis was determined 24 h later. *Panel B.* LNCaP cells were infected with a dominant-negative PKCε AdV and 24 h later treated with PMA. *Inset*, DN-PKCε expression. Apoptosis was determined 24 h later. *Panel C.* Apoptosis measured in LNCaP, the LNCaP variant C4, and DU145 cells, 24 h after treatment with PMA (100 nM, 1 h). *Inset*, Western blot showing the depletion of PKCs. Data are expressed as mean ± S.E. of 3 independent experiments.

Figure 5. PKCε overexpression inhibits PMA-induced apoptosis in LNCaP cells. LNCaP cells were infected with different MOIs of PKCε adenovirus (AdV) or control LacZ AdV. After

48 h, cells were treated with PMA (100 nM, 1 h) or vehicle, and the incidence of apoptosis was determined 24 h later. Expression of PKCε is shown by Western blot using a specific anti-PKCε antibody.

Figure 6. Global analysis of PMA-regulated gene expresssion in LNCaP cells. LNCaP cells were treated with PMA (100 nM, 1 h). RNA was prepared at different times and gene expression analyzed with Affymetrix U133A v 2.0 microarray. *Panel A.* Flow chart of data evaluation and selection criteria. *Panel B.* Heat map of PMA-regulated genes. *Panel C.* Gene ontology analysis of PMA-regulated genes at 4 h.

Figure 7. Androgens regulate TNFα and TRAIL mRNA release by PMA. LNCaP cells were grown in normal medium, steroid-depleted medium, or steroid-depleted medium supplemented with R1881 (1 nM) for 48 h and then treated with either 100 nM PMA (+ *PMA*) or vehicle (- *PMA*) for 1 h. TNFα (*Panel A*) and TRAIL levels (*Panel B*), as determined by ELISA in CM collected 24 h after PMA or vehicle treatment. Each sample was run by triplicates and results are presented as mean ± S.D. (n=3). Two additional experiments gave similar results. *CM-PMA*, conditioned medium from PMA-treated cells; *CM-Veh*, conditioned medium from vehicle-treated cells.

Figure 8. AR RNAi inhibits PMA induction of TNFα and TRAIL and apoptosis. LNCaP cells growing in normal medium were transfected with either an RNAi duplex for AR or a control (*C*) duplex using the Amaxa Nucleofector, and 48 h later treated for 1 h with either 100 nM PMA or vehicle. For mRNA determinations, RNA was extracted 3 h after treatment. For cytokine determinations, CM was collected 24 h after treatment. *Panel A.* Representative Western blot showing AR depletion and PKCδ down-regulation in AR-depleted cells 48 h after RNAi transfection. Expression levels, relative to control RNAi, have been determined by densitometry and are shown below each corresponding Western blot. *Panel B.* Apoptotic effect of CM-PMA collected from LNCaP cells from cells subjected to AR or control RNAi. The percentage of apoptotic cells was determined by DAPI staining 24 h after addition of CM-PMA. *Panels C and D*. TNFα and TRAIL mRNA levels were determined by real-time PCR 3 h after PMA treatment in LNCaP cells subject to either AR or control RNAi. Results are normalized to endogenous GAPDH mRNA levels and expressed as fold-increase relative to those in control cells treated with vehicle. *Panel E and F*. TNFα and TRAIL levels in CM-Veh or CM-PMA collected from LNCaP cells subjected to AR or control RNAi, as determined by ELISA. In all cases, a representative experiment is shown and results are presented as mean ± S.D. of triplicate samples. Similar results were obtained in two additional experiments. *CM-PMA,* conditioned medium from PMA-treated cells; *CM-Veh,* conditioned medium from vehicle-treated cells.

Figure 9. Androgen depletion and AR RNAi impair the ability of CM-PMA to activate p38 MAPK and JNK. *Panel A.* LNCaP cells growing in normal medium were transfected with RNAi duplexes for either AR or a control (*C*) duplex using the Amaxa Nucleofector, and 48 h later treated for different times with 100 nM PMA . *Panel B.* LNCaP cells were grown in normal medium, steroid-depleted medium, or steroid-depleted medium supplemented with R1881 (1 nM) for 48 h, and then treated with either 100 nM PMA (+ *PMA*) or vehicle (- *PMA*) for 1 h. For *Panels A* and *B,* CM was collected 24 h after treatment and added to LNCaP cells. Cell extracts were prepared and subjected to Western blot analysis using the antibodies indicated in the

figures. Phospho-38 and phospho-JNK levels, normalized to the corresponding total levels, were determined by densitometry. Values were expressed as fold-increase relative to t = 0 (*Panel A*) or to cells growing in normal medium and untreated with PMA (*Panel B*). Values are shown under each corresponding Western blot. Similar results were observed in 3 independent experiments.

Figure 1

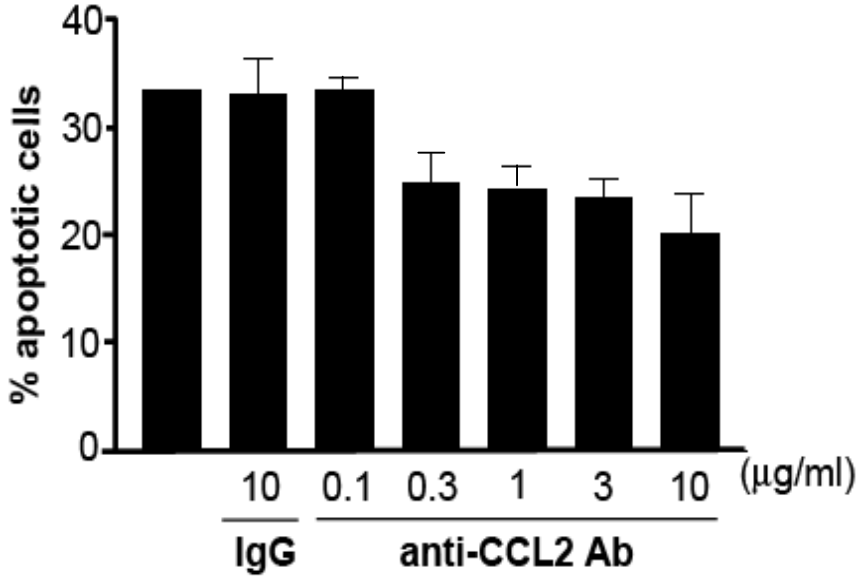

% apoptotic cells
40
30
20
10
0
10
0.1
0.3
1
3
10
(μg/ml)
IgG
anti-CCL2 Ab

Figure 2

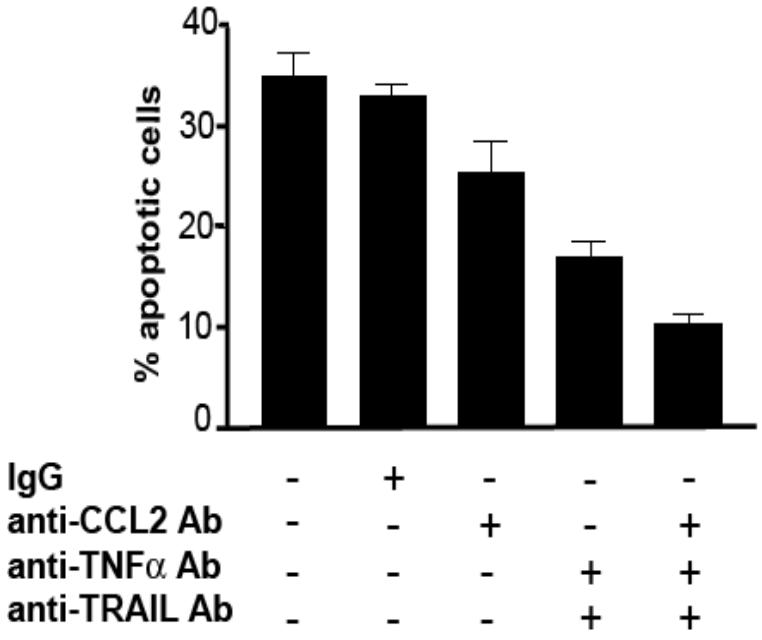

40
30
20
10
0
% apoptotic cells
IgG - + - - -
anti-CCL2 Ab - - + - +
anti-TNFα Ab - - - + +
anti-TRAIL Ab - - - + +

Figure 3

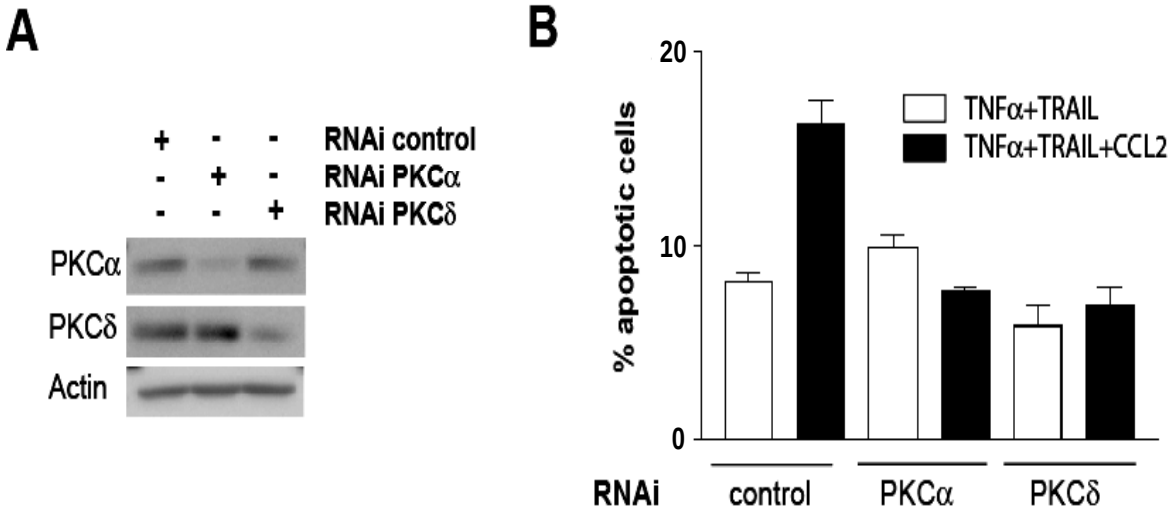

A
+ - - RNAi control
- + - RNAi PKCα
- - + RNAi PKCδ
PKCα
PKCδ
Actin
B
% apoptotic cells
20
10
0
TNFα+TRAIL
TNFα+TRAIL+CCL2
RNAi
control
PKCα
PKCδ

Figure 4

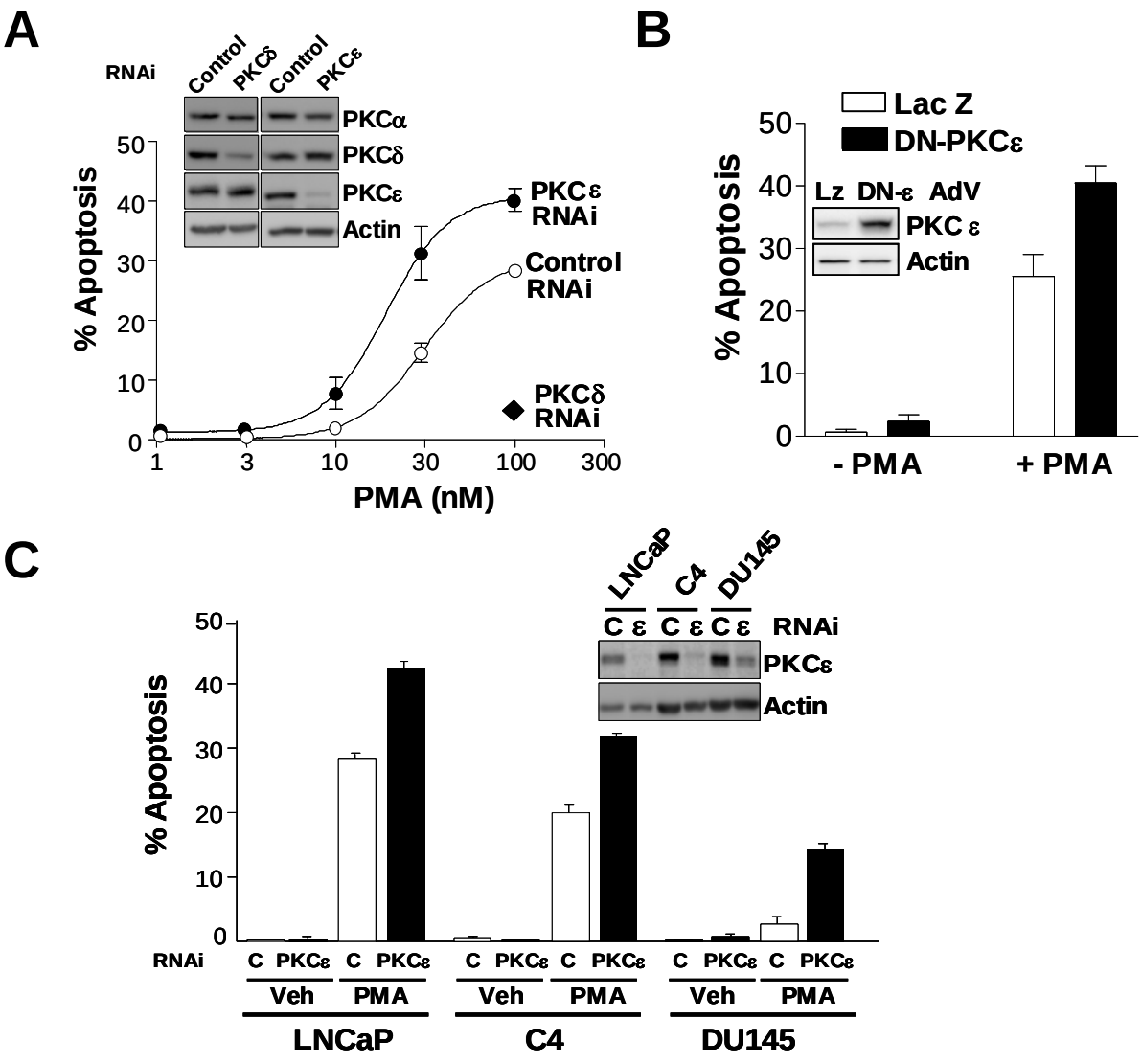

A
RNAi
Control
PKCδ
Control
PKCε
PKCα
PKCδ
PKCε
Actin
% Apoptosis
PKCε RNAi
Control RNAi
PKCδ RNAi
PMA (nM)
B
Lac Z
DN-PKCε
Lz DN-ε AdV
PKC ε
Actin
% Apoptosis
- PMA
+ PMA
C
LNCaP
C4
DU145
RNAi
PKCε
Actin
% Apoptosis
Veh
PMA
LNCaP
C4
DU145

Figure 5

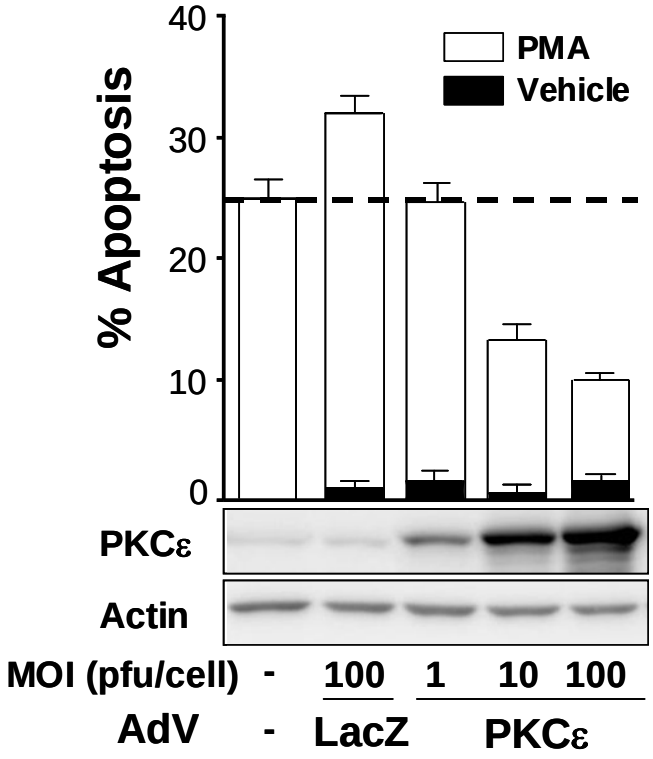

40
30
20
10
0
% Apoptosis
PMA
Vehicle
PKCε
Actin
MOI (pfu/cell) - 100 1 10 100
AdV - LacZ PKCε

Figure 6

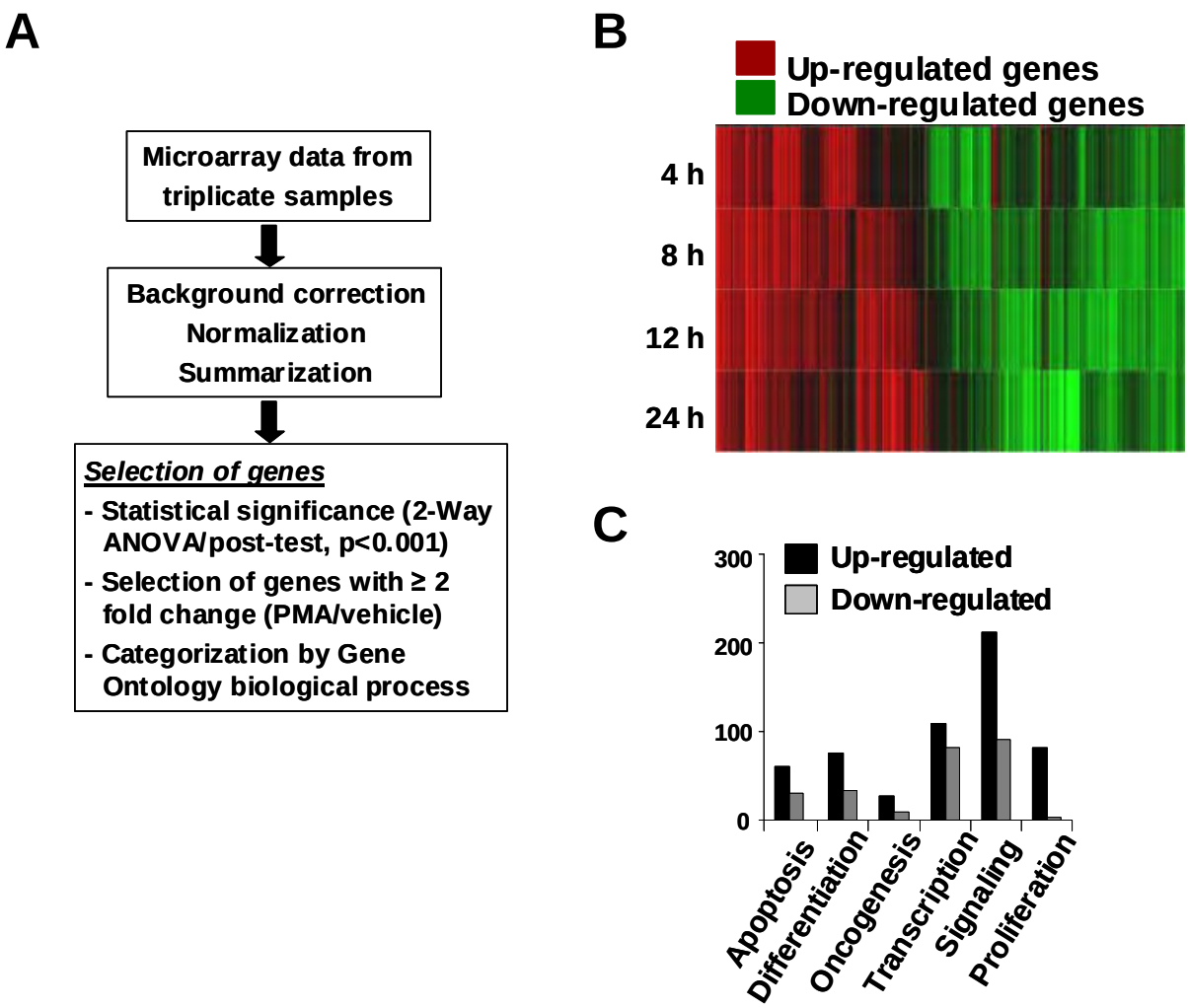

A
Microarray data from triplicate samples
Background correction
Normalization
Summarization
Selection of genes
- Statistical significance (2-Way ANOVA/post-test, p<0.001)
- Selection of genes with ≥ 2 fold change (PMA/vehicle)
- Categorization by Gene Ontology biological process
B
Up-regulated genes
Down-regulated genes
4 h
8 h
12 h
24 h
C
300
200
100
0
Up-regulated
Down-regulated
Apoptosis
Differentiation
Oncogenesis
Transcription
Signaling
Proliferation

Figure 7

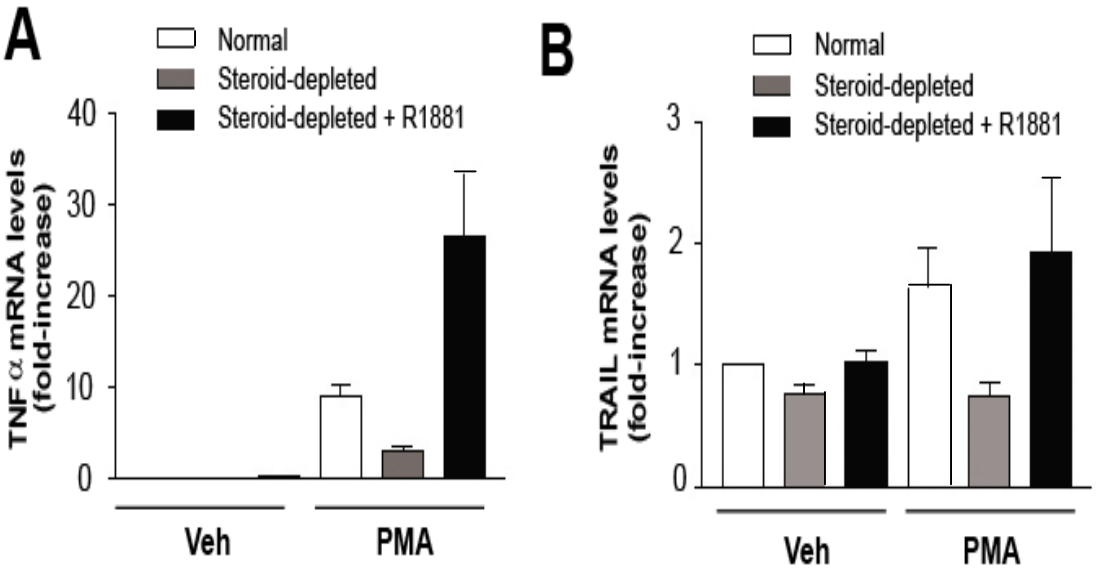

A
Normal
Steroid-depleted
Steroid-depleted + R1881
TNF α mRNA levels
(fold-increase)
40
30
20
10
0
Veh
PMA
B
Normal
Steroid-depleted
Steroid-depleted + R1881
TRAIL mRNA levels
(fold-increase)
3
2
1
0
Veh
PMA

Figure 8

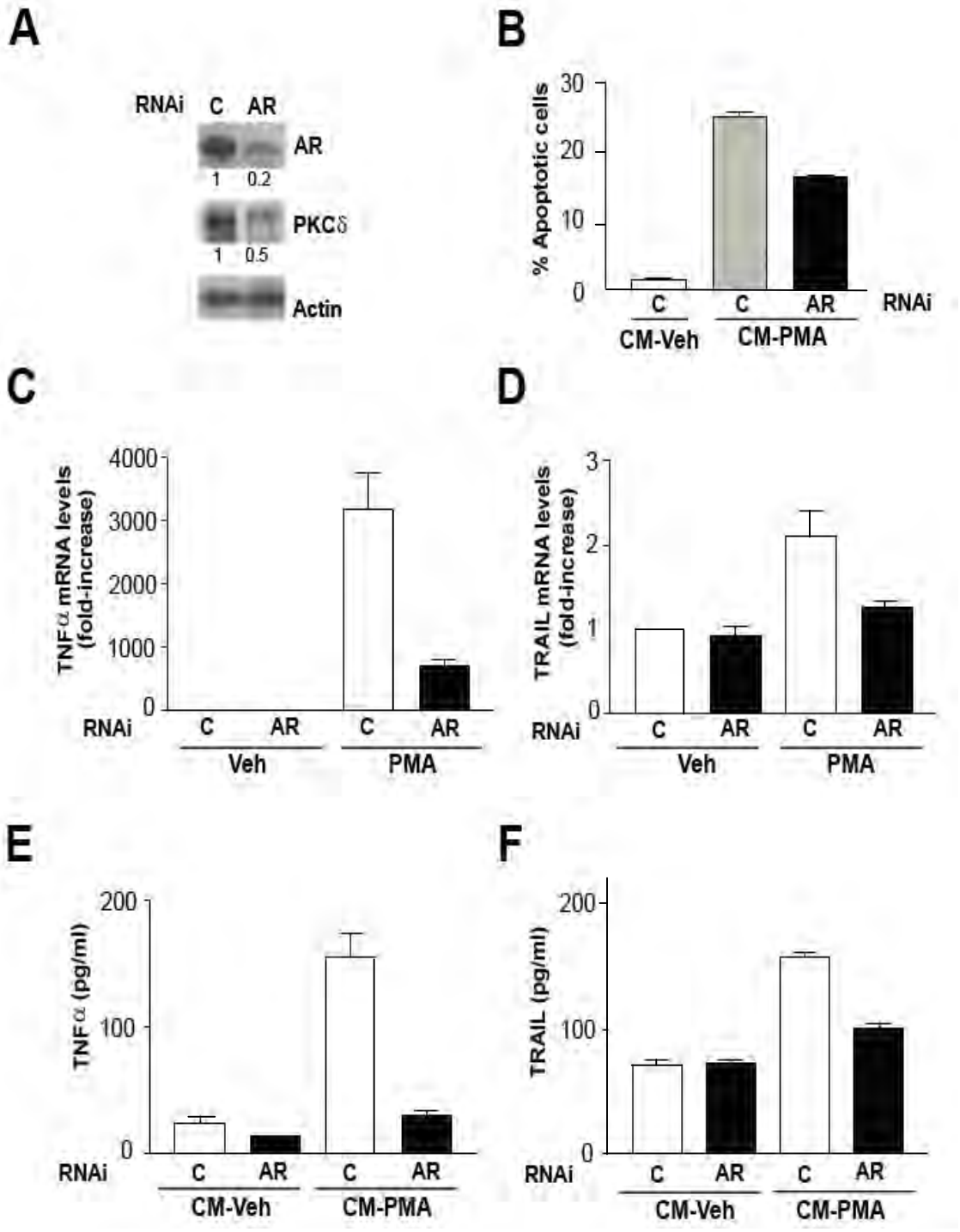
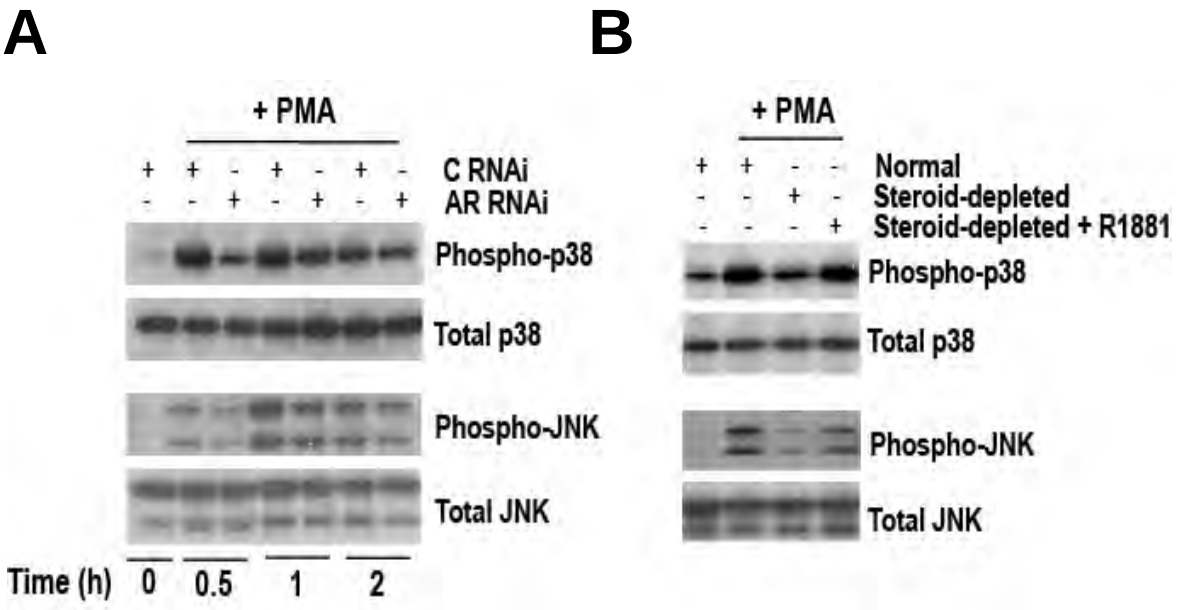

A
RNAi C AR
AR
1 0.2
PKCδ
1 0.5
Actin
B
% Apoptotic cells
30
20
10
0
C C AR RNAi
CM-Veh CM-PMA
C
TNFα mRNA levels (fold-increase)
4000
3000
2000
1000
0
RNAi C AR C AR
Veh PMA
D
TRAIL mRNA levels (fold-increase)
3
2
1
0
RNAi C AR C AR
Veh PMA
E
TNFα (pg/ml)
200
100
0
RNAi C AR C AR
CM-Veh CM-PMA
F
TRAIL (pg/ml)
200
100
0
RNAi C AR C AR
CM-Veh CM-PMA

Figure 9


A
+ PMA
C RNAi
AR RNAi
Phospho-p38
Total p38
Phospho-JNK
Total JNK
Time (h) 0 0.5 1 2
B
+ PMA
Normal
Steroid-depleted
Steroid-depleted + R1881
Phospho-p38
Total p38
Phospho-JNK
Total JNK